added, the volume completed to 200 ml., and the resulting solution maintained at 35°. Samples were taken at intervals, the periodate consumed and the formic acid produced were titrated in the usual way. Formaldehyde was determined according to Reeves.[18] Results are given in the table below.

(18) R. E. Reeves, *J. Am. Chem. Soc.*, **63**, 1476 (1941).

Acknowledgments. We thank Mr. Joseph F. Alicino, from The Squibb Institute for Medical Research, New Brunswick, N. J., for the microanalysis.

BUENOS AIRES, ARGENTINA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITÉ DE MONTRÉAL]

Preparation of L-Cystinyl and L-Cysteinyl Peptides Through Catalytic Hydrogenation of Intermediates

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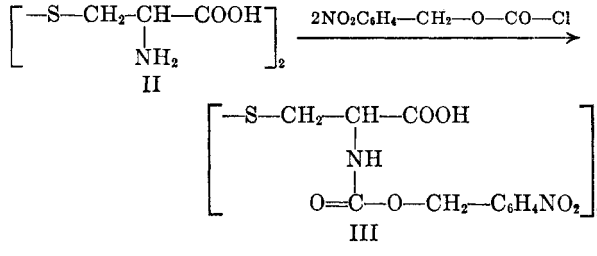
Received February 4, 1957

The preparation of peptides containing cystine or cysteine by the general method of catalytic hydrogenolysis of intermediates becomes possible if the classical carbobenzoxyl group is replaced by the more labile *p*-nitrocarbobenzoxyl radical to cover uncondensed α-amino groups. The hydrogenation procedure can be arrested at the cystine stage or allowed to proceed to cysteine. Likewise, if the *p*-nitrobenzyl radical is used to cover the thio group of cysteine, it can be removed by catalytic hydrogenation, whereas *S*-benzylcysteine intermediates are only cleaved by sodium in liquid ammonia. The preparation of L-cystinyldiglycine, L-cystinyldi-L-phenylalanine, and of L-cysteinyl-L-phenylalanine are proposed as typical examples.

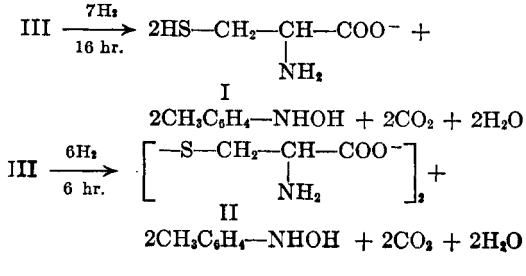
Cystine has been reduced to cysteine by catalytic hydrogenation with palladium.[1,2] However, in attempting to hydrogenate dicarbobenzoxycystine according to the general Bergmann and Zervas procedure,[3] White[4] found that no reduction took place. It has indeed frequently been observed and it is now accepted that the efficiency of palladium or of platinum as a catalyst is sharply reduced whenever sulfur is present in the form of a dithio linkage as in cystine, of a thiol group as in cysteine, or of a thioether as in *S*-benzylcysteine. The exact limits of this incompatibility have not, however, been explored since Siffered and du Vigneaud's alternate method[5] of reduction of *N*-carbobenzoxyl and *S*-benzyl derivatives of cystine or of cysteine with sodium in liquid ammonia was adopted at an early period for the introduction of these two amino acids in synthetic peptides.

The authors have previously shown[6] that α-*p*-nitrocarbobenzoxy-L-arginyl derivatives are easily reduced to L-arginyl peptides by hydrogen at atmospheric pressure in the presence of palladium on carbon. In view of the increased ease of removal of the *p*-nitrocarbobenzoxyl radical which was noted as a result of labilization by the strong inductive effect of the nitro group, the authors were brought to use this radical to cover the basic amino groups of cystine and to investigate the possibility of its removal by catalytic hydrogenation under conditions where the carbobenzoxyl group is stable.

Di(*p*-nitrocarbobenzoxy)-L-cystine III was prepared by condensing *p*-nitrocarbobenzoxyl chlorocarbonate with L-cystine II in tetrahydrofuran or dioxane. The disubstituted cystine was submitted

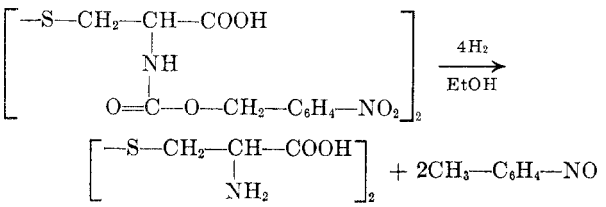


to catalytic hydrogenation at room temperature under atmospheric pressure in the presence of palladium black on carbon. In aqueous medium, when the disubstituted cystine was dissolved as the sodium salt, absorption of hydrogen proceeded rapidly at first, and continued until an equivalent of seven moles were consumed in about 16 hr. The products of reduction consisted of L-cysteine I and *p*-tolylhydroxylamine. If the hydrogenation process was arrested when 6 moles of hydrogen had been absorbed (about 6 hr.), L-cystine II and *p*-tolylhydroxylamine were obtained.

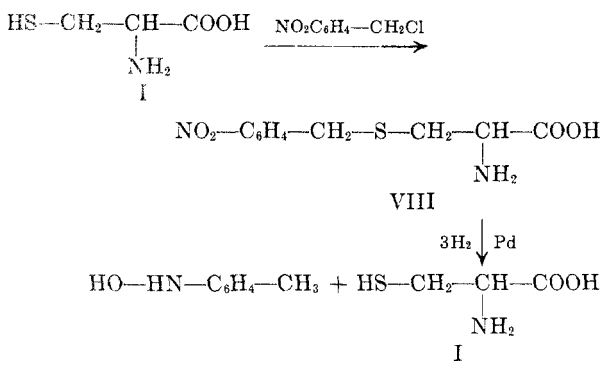


(1) M. Bergmann and G. Michalis, *Ber.*, **63B**, 987 (1930).
(2) E. Kavanagh Kevin, *J. Am. Chem. Soc.*, 65, 2721 (1942).
(3) M. Bergmann and L. Zervas, *Ber.*, 65, 1192 (1932).
(4) J. White, *J. Biol. Chem.*, **106**, 141 (1934).
(5) H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1953).
(6) C. Berse and L. Piché, *J. Org. Chem.*, **21**, 808 (1956).

When the disubstituted cystine was hydrogenated in ethanol, four moles of hydrogen only were absorbed. Cystine was afforded in quantitative yield (96%) while *p*-nitroso-toluene was also probably formed. Reduction to cysteine was not then possible, cystine precipitating out of solution as it is formed.



The authors have found that *S*-*p*-nitrobenzylcysteine is similarly reduced by catalytic hydrogenation, whereas *S*-benzylcysteine is cleaved only by reduction with sodium in liquid ammonia.[4] *S*-*p*-nitrobenzyl-L-cysteine VIII was prepared by condensing L-cysteine I with *p*-nitrobenzyl chloride; it was suspended in 1*N* HCl or dissolved in alcohol and hydrogenated at room temperature, under atmospheric pressure over 10% palladium on carbon. Three moles of hydrogen were absorbed and cysteine was obtained.



L-cysteinyl dipeptides or symmetrical L-cystinyl tripeptides were accordingly prepared by first condensing di(*p*-nitrocarbobenzoxy)-L-cystine III with the ethyl ethers of glycine and of phenylalanine by the mixed anhydride method.[7]

The resulting covered ester of a symmetrical tripeptide IV was saponified with a slight excess of sodium hydroxide in the cold; the dithio linkage and the peptide bond remain untouched, and the resulting dicarboxylic structure V is hydrogenated as indicated for III. Partial hydrogenation, arrested when four moles of hydrogen have been absorbed, yields the L-cystinyl symmetrical tripeptides VI. Or V can be fully hydrogenated to provide the L-cysteinyl dipeptides VII. Oxidation of VII with air in alkaline aqueous solution affords a return to VI; cystinyl derivatives thus obtained were found to be identical to those prepared directly by partial reduction.

The labilization of the classical carbobenzoxyl and benzyl groups produced by the introduction of a *p*-nitro radical affords the possibility of reducing peptide intermediates containing cystinyl or cysteinyl residues, in which part of the structure is not amenable to reduction with sodium in liquid ammonia.

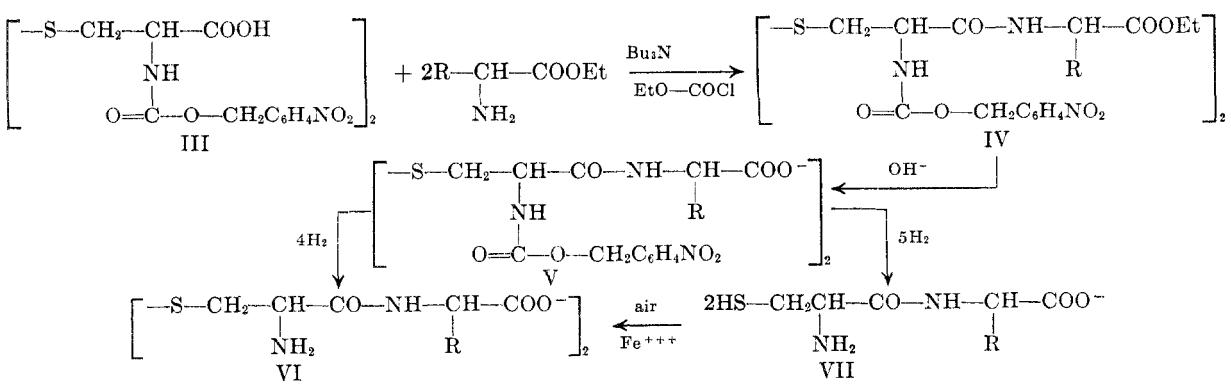
EXPERIMENTAL

Melting points, unless otherwise indicated, have been determined in semicapillary tubes and are uncorrected. In some cases where the materials decomposed, instantaneous melting points have been determined on the Maquenne block.

Di-p-nitrocarbobenzoxy-L-cystine. L-cystine (12 g., 0.05 mole) was dissolved in 50 ml. of 2*N* NaOH and 20 ml. of purified dioxane was added. The solution was stirred vigorously and cooled to 0°; *p*-nitrocarbobenzoxyl chloride (33 g., 0.15 mole) dissolved in 150 ml. of dioxane with 100 ml. of 2*N* NaOH was added in 5 portions (30 min.). Stirring was continued for 0.5 hr. after the addition of all reactants. The mixture was allowed to come to room temperature and 300 ml. of water was added. The resulting alkaline solution was washed twice with ethyl acetate and acidified to Congo Red with 1*N* HCl. The product was extracted 4 times with 100-ml. portions of ethyl acetate. After evaporation of the ethyl acetate, the crude product, an oil, was crystallized from nitromethane. Yield: 22.2 g. (74%); m.p. 110–111°; $[\alpha]_D^{25}$ – 126.4 (*c*, 2.0 in ethanol).

Anal. Calcd. for $C_{22}H_{22}N_4O_{12}S_2$: C, 44.16, H, 3.70; N, 9.36. Found: C, 44.16; H, 3.73; N, 9.28.

Hydrogenation of di-p-nitrocarbobenzoxy-L-cystine. (a) *In aqueous solution.* Di-*p*-nitrocarbobenzoxy-L-cystine (0.599 g.,



R = H (glycine)
R = CH_2-C_6H_5 (L-phenylalanine)

(7) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

1mmole) was dissolved in .05*N* NaOH (40 ml.) and was hydrogenated at room temperature and at atmospheric pressure over 10% palladium on carbon (250 mg.). After approximately 6 hr., 6 moles of hydrogen were absorbed. The catalyst and *p*-tolylhydroxylamine were separated by filtration and the solution was acidified to *p*H 5 with HCl. By concentration, L-cystine crystallized in practically quantitative yield.

When hydrogenation was allowed to continue, an extra mole of hydrogen was absorbed in the course of 10 hr. After filtration, the solution was neutralized with HCl, concentrated to a low bulk under reduced pressure, and the residue was dissolved in warm 5*N* HCl. On cooling, the hydrochloride of L-cysteine crystallized. Yield, 284 mg. (90%).

p-Tolylhydroxylamine was extracted with ether from the mother liquors and recrystallized from a mixture of benzene and petroleum ether. It melted at 93–94°.

(b) *In alcohol.* Di-*p*-nitrocarbobenzoxy-L-cystine (0.599 g., 1 mmole) was dissolved in 95% ethanol (40 ml.) and was hydrogenated as above. After approximately 6 hr., 4 moles of hydrogen were absorbed, and the reaction ceased. It was found that cystine precipitated out of solution as it was formed. Filtration separated the catalyst and cystine; cystine was extracted with 20 ml. of *N* NaOH. After filtration, the solution was acidified with HCl to *p*H 5. By concentration, L-cystine crystallized. Yield, 230 mg. (96%).

The mother liquor contained a yellow, unstable product which was considered to be *p*-nitrosotoluene; no formal characterization was attempted.

Ethyl di-p-nitrocarbobenzoxy-L-cystinyl diglycinate. Di-*p*-nitrocarbobenzoxy-L-cystine (4.8 g., 0.008 mole) was dissolved in tetrahydrofuran, (30 ml.) previously dried over sodium, and tri-*n*-butylamine (3.8 ml., 0.016 mole) was added. The mixture was cooled with ice-salt mixture and ethyl choroformate (1.5 ml., 0.016 mole) was added. The mixture was stirred for 15 min. After this time, a solution of glycine ethyl ester hydrochloride (2.23 g., 0.016 mole) and tri-*n*-butylamine (3.8 ml., 0.016 mole) in chloroform (20 ml.) was added. The mixture was stirred for 1 hr. at room temperature and the solvent was evaporated *in vacuo* at 50–60°. The residue, a thick oil, was dissolved in chloroform (100 ml.) and this solution was washed with 1*N* HCl, water, 5% aqueous bicarbonate and water, and then dried over anhydrous sodium sulfate. The solvent was evaporated *in vacuo* and the residue was recrystallized from nitromethane. Yield, 4.4 g., (75%); m.p. 160–161, $[\alpha]_D^{25}$ − 76.6 (*c*, 1.1, in acetone).

Anal. Calcd. for $C_{30}H_{36}N_6O_{14}S_2$: C, 46.87; H, 4.72; N, 10.82. Found: C, 46.84; H, 4.68; N, 10.94.

Ethyl di-p-nitrocarbobenzoxy-L-cystinyl-di-L-phenylalanate. This compound was prepared from di-*p*-nitrocarbobenzoxy-L-cystine (2.4 g.) and phenylalanine ethyl ester hydrochloride (1.83 g.) as above, and recrystallized from nitromethane; yield, 3.1 g. (81%); m.p. 173–174°; $[\alpha]_D^{25}$ − 37.8 (*c*, 0.6 in acetone).

Anal. Calcd. for $C_{44}H_{48}N_6O_{14}S_2$: C, 55.68; H, 5.09; N, 8.85. Found: C, 55.65; H, 5.09; N, 8.80.

Di-p-nitrocarbobenzoxy-L-cystinyldiglycine. Ethyl di-*p*-nitrocarbobenzoxy-L-cystinyldiglycinate (2 g.) was dissolved in dioxane (75 ml.) and NaOH 0.1*N* (70 ml.) was added in two portions during the course of 1 hr. The mixture was stireed at 0–5° for the first hour and at room temperature for 0.5 hr. Four hundred ml. of water was added to the solution. The solution was extracted with ethyl acetate, and the aqueous layer was acidified to Congo Red with concentrated hydrochloric acid. The resulting crystalline product was collected, and was recrystallized from nitromethane. Yield, 1.75 g., (95%); m.p. 111–113°; $[\alpha]_D^{25}$ − 79.8 (*c*, 1.3 in acetone).

Anal. Calcd. for $C_{28}H_{28}N_6O_{14}S_2 \cdot H_2O$: C, 43.01; H, 4.14; N, 11.50. Found. C, 43.02; H, 4.29; N, 11.32.

Di-p-nitrocarbobenzoxy-L-cystinyl-di-L-phenylalanine. Ethyl di-*p*-nitrocarbobenzoxy-L-cystinyldiphenylalanate (2 g.) was dissolved in dioxane (70 ml.) and 0.2*N* NaOH (23 ml.) was added in two portions during the course of 1 hr. The mixture was stirred at 0–5° for the first hour and at the room temperature for 0.5 hr. The solution was acidified to Congo Red and extracted with ether. The ether layer was dried over anhydrous sodium sulfate. The solvent was evaporated *in vacuo* and the residue was recrystallized from dioxane (minimum)-ether. Yield, 1.6 g., (85%); m.p. 118–120°; $[\alpha]_D^{25}$ − 49.1 (*c*, 2.5 in acetone).

Anal. Calcd. for $C_{40}H_{40}O_{14}N_6S_2$. C, 53.80; H, 4.51; N, 9.41. Found: C, 53.91; H, 4.85; N, 9.20.

L-Cystinyldiglycine. Di-*p*-nitrocarbobenzoxy-L-cystinyldiglycine (7.30 g.) was dissolved in 95% ethanol (25 ml.). The compound was hydrogenated for 8 hr. at room temperature and at atmospheric pressure over 10% palladium on carbon (250 mg.). Filtration separated the catalyst and L-cystinyl diglycine; L-cystinyl-diglycine was extracted with 20 ml. of *N* NaOH. The solution was neutralized to litmus with HI (15%). After nltration, the solvent was concentrated *in vacuo* at 40–50°; the product then was precipitated with ethanol. The peptide was recrystallized from water-ethanol. Yield: 0.320 g. (90%), m.p. 210° dec. (literature[8] m.p. 210° dec.) $[\alpha]_D^{25}$ − 84.2 (*c*, 0.5 in 1*N* HCl).

Anal. Calcd. for $C_{10}H_{18}N_4O_6S_2$. C, 33.89; H, 5.12; N, 15.79. Found. C, 34.39; H, 5.27; N, 15.40.

L-Cystinyl-di-L-phenylalanine (2 HCl). Di-*p*-nitrocarbobenzoxy-L-cystinyldi-L-phenylalanine (0.893 g., 1 mmole) was hydrogenated in 95% ethanol (30 ml.) in the manner described above; L-cystinyldi-L-phenylalanine was extracted with 20 ml. of *N* NaOH. The solution was neutralized to litmus with HI (15%). After filtration, the solvent was evaporated *in vacuo* at 40–50° and the residue was washed with ethanol in order to remove sodium iodide. The solution of the peptide in *N*-hydrochloric acid at about 5% concentration slowly deposited crystals; addition of an equal volume of concentrated hydrochloric acid and chilling gave the hydrochloride. Yield: 0.530 g. (87%), m.p. 256° dec. $[\alpha]_D^{25}$ − 57.3 (*c*, 0.8 in 1*N* HCl).

Anal. Calcd. for $C_{24}H_{32}N_4O_6Cl_2S_2$: C, 47.43; H, 5.31; N, 9.22. Found: C, 47.45; H, 5.38; N, 9.23.

L-Cysteinyl-L-phenylalanine. L-cystinyldi-L-phenylalanine hydrochloride (0.608 g., 1 mmole) was dissolved in 1*N* NaOH (40 ml.). The compound was hydrogenated for 12 hr. at room temperature and atmospheric pressure over 10% palladium on carbon (250 mg.). The catalyst was then removed by filtration and the filtrate was concentrated and adjusted to about *p*H 4.8 by addition of concentrated HCl. Crystals were obtained by adding ethanol cautiously to a small portion of the solution, and on inoculation of the main bulk, crystallization rapidly set in. After keeping overnight, the precipitate was collected and dried. Yield: 0.485 g., (91%), m.p. > 300° (dec.) $[\alpha]_D^{25}$ − 8.9 (*c*, 2, 1*N* HCl).

Anal. Calcd. for $C_{12}H_{16}N_2O_3S$: C, 53.71; H, 6.01; N, 10.44. Found: C, 53.67; H, 5.99; N, 10.40.

S-p-Nitrobenzyl-L-cysteine. L-cysteine hydrochloride (3.14 g., 20 mmoles) was dissolved in 1*N* NaOH (60 ml., 60 mmoles). The solution was stirred vigorously and *p*-nitrobenzyl chloride (1.71 g., 20 mmoles) dissolved in dioxane (30 ml.) was added at 0° during 30 min., in five approximately equal portions. The reaction mixture was then stirred at room temperature for 30 min. The resulting alkaline solution was washed twice with ether, acidified to litmus with concentrated HCl and the organic solvent was evaporated *in vacuo*. *S-p*-nitrobenzyl-L-cystine hydrate precipitated within a few hours and was recrystallized from hot water. Yield: 3.2 g. (60%); m.p. 233–234°. For analysis the product was dried for 18 hr. *in vacuo* over P_2O_5 at 78°.

Anal. Calcd. for $C_{10}H_{12}N_2O_4S \cdot H_2O$: C, 43.78; H, 5.14; N, 10.21. Found: C, 43.73; H, 5.09; N, 10.22.

Ethyl ester, m.p. 172–173°. $[\alpha]_D^{25}$ + 27.3 (*c*, 1.06 in ethanol 95%).

Anal. Calcd. for $C_{12}H_{16}O_4N_2S \cdot HCl$: C, 44.93; H, 5.02; N, 8.73. Found. C, 44.80; H, 5.31; N, 8.69.

(8) H. S. Loring and V. du Vigneaud, *J. Biol. Chem.*, **111**, 385 (1935).

Catalytic reduction of S-p-nitrobenzyl-L-cysteine. S-p-nitrobenzyl-L-cysteine H_2O (0.548 g.) was dissolved in ethanol (40 ml.) and 1*N* HCl (20 ml.). The compound was hydrogenated for 3 hr. at room temperature and atmospheric pressure over 10% palladium on carbon (138 mg.). The catalyst was separated by filtration. The filtate gave the positive nitroprusside test for sulfhydryl. The product was precipitated as mercaptide with Hopkin's reagent. After 24 hr., the mercaptide was filtered and washed with cold water. The product was suspended in water (20 ml.) then stirred and saturated with H_2S, and the mercury sulfide was separated by filtration. The filtrate was made alkaline with sodium hydroxide and a small crystal of copper sulfate was added. Air was bubbled through the solution until the violet color disappeared (2 hr.). The solution was decolorized with charcoal, filtered, and neutralized with HCl. Crystallization soon began. After standing for 2 hr. at room temperature, the product was filtered, washed with cold water, alcohol and ether. For recrystallization the product was dissolved in 1*N* NaOH, then neutralized with 1*N* HCl. Yield: 96 mg. (40%), m.p. 255–260° (dec.) $[\alpha]_D^{25}$ − 225° (*c*, 1.04 in 1*N* HCl).

Acknowledgment. The authors wish to express their thanks for the aid of Rougier Frères Ltée who supported their research.

MONTREAL, QUE.
CANADA

Isolation of Vitamin Dm and Vitamin D_3 from the Irradiation Products Obtained from Sterols of the Mussel, *Modiolus Demissus*, Dillwyn[1]

HAROLD G. PETERING[2]

Received November 3, 1954

A new vitamin D named vitamin Dm has been isolated from the irradiation products of the sterols derived from the ribbed mussel, *modiolus demissus*, Dillwyn. It has been characterized by the physical and chemical properties of the crystalline dinitrobenzoate. Its biological efficacy has been shown to be about 30,000,000 U.S.P. or A.O.A.C. units per g. of resin, and to be equal for rats and chicks.

The new compound is distinct from the known purified and crystalline vitamins D. It exists side by side in the ultraviolet irradiation products of the mussel sterols with vitamin D_3. A method for separating these two compounds as the dinitrobenzoates is described.

According to available reports only three compounds having vitamin D activity have been obtained in crystalline form or as crystalline derivatives. Calciferol, or vitamin D_2, was first obtained by Askew *et al.*,[3] and later also by Windaus and coworkers.[4] Vitamin D_3 was first isolated by Brockmann[5,6] from fish liver oils as the crystalline dinitrobenzoate, from which Brockmann and Busse[7,8] prepared the crystalline vitamin. Later the same compound was obtained by Windaus, Schenck, and von Werder[9] and by Schenck[10] from the irradiation products of 7-dehydrocholesterol both as crystalline esters and as the free alcohol. Finally, vitamin D_4 was obtained in pure form as the free alcohol and as an ester by Windaus and Trautmann[11] from the irradiation products of 22,23-dihydroergosterol.

The biological efficacy of these three vitamins D are established with reasonable accuracy because of the availability of the pure compounds. The existence of other vitamins D from time to time has been reported, but in every instance the evidence rests on comparative biological assay and little actually is known of the structure of these compounds or of their real efficacy and physiological function.

This report deals with the isolation of a fourth vitamin D, vitamin Dm, from the irradiation products of the sterols obtained from the mussel, *modiolus demissus*, Dillwyn, and also describes the separation of this compound from vitamin D_3 which is also found in the irradiation products of the mussel sterols.

Petering and Waddell[12] recently described the isolation and characterization of a new provitamin Dm which was isolated from the same ribbed mussel, *modiolus demissus*, Dillwyn, and which appears to be a C_{29} sterol. In the early stages of that investigation of the sterols of the ribbed mussel, it did not seen likely that a sufficient sample of the purified provitamin Dm would be available to permit a careful study of its properties and also allow for the irradiation of a portion for the isolation of the corresponding vitamin D. Therefore, it was de-

(1) This work was carried out in the Biological Laboratory of the E. I. du Pont de Nemours & Co., Inc.

(2) Present Address: The Upjohn Co., Kalamazoo, Mich.

(3) F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. K. Callow, J. S. L. Philpot, and T. A. Webster, *Proc. Royal Soc. (Lond.)*, **109B**, 488 (1932).

(4) A. Windaus, A. Luttringhaus, and M. Deppe, *Ann.*, **489**, 252 (1931); A. Windaus, and A. Luttringhaus, *Z. Physiol. Chem.*, **203** 70 (1931).

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(7) H. Brockmann, and A. Busse, *Z. Physiol. Chem.*, **249**, 176 (1937).

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(9) A. Windaus, F. Schenck, and F. v. Werder, *Z. Physiol. Chem.*, **241**, 100 (1936).

(10) F. Schenck, *Naturwissenschaften*, **25**, 159 (1937).

(11) A. Windaus, and G. Trautmann, *Z. Physiol. Chem.*, **247**, 185 (1937).

(12) H. G. Petering, and J. Waddell, *J. Biol. Chem.*, **191**, 765 (1951).